PATENT
2503-1170

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of

Daniele CALISTRI et al. Conf. 1643

Application No, 10/547,669 Group 1637

Filed September 2, 2005 Examiner M. Staples

METHOD FOR THE IDENTIFICATION OF COLORECTAL TUMORS

DECLARATION UNDER RULE 132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Daniele Calistri, hereby declare as follows:

My relevant background and experience are set forth on the c.v. previously filed with the Declaration filed of March 14, 2008.

I make this declaration in support of the present application, and to provide further evidence in rebuttal of several contentions set forth in the outstanding Official Action. This declaration includes a sample concordance correlation coefficient and a summary of sensitivity/specificity data in a single table, which were not included the Declaration filed of March 14, 2008

I do not believe that any of the publications cited in the Office Action mailed April 16, 2009, taken alone or in combination, disclose or suggest the claimed invention. In particular, I do not believe that any of the publications disclose or suggest the primers recited in the claims.

Table 1 shows the results, in terms of FL-DNA (Fluorescence long DNA) values expressed as nanograms (see also patent description), obtained by two different approaches:

i) a method that utilizes the primers recited in the claimed invention, and

ii) a method that utilizes a series of primers designed in the same genomic regions. The results show that the two sets of data are not comparable in many cases, indicating that the use of a selected series of primers determine different FL-DNA values. This difference is also indicative of a different ability to discriminate between cancer patients and healthy subjects. In fact, the aim of the FL-DNA method is to identify cancer patients with the highest sensitivity and specificity possible.

Table 2 shows the sensitivity and specificity of the two different approaches. Using the primers cited in our claims, i.e., "claimed" in the Table 2, it is possible to obtain good sensitivity and specificity with different cut-offs (see, for example, 10-15 or 20 ng cut-offs). Conversely, using other primers in the same genomic region as the claimed primers ("new" in Table 2), sensitivity and specificity are rarely high for the same cut-off, and good sensitivity tends to correlate with poor specificity, and vice-versa. This problem does not allow one to identify an accurate (high sensitivity and specificity) cut-off for colorectal cancer detection.

Table 1

Results of FL-DNA analysis using different primers

Tumor no.	FL-DNA (ng) "Claimed" primers	"New" primers
1	34	33
2	41	9
3	19	12
4	42	12
5	10	10
6	9	3
7	35	3
8	21	8
9	96	6
10	77	11
Healthy donor no.		
1	9	6
2	13	9
3	13	7
4	14	13
6	4	13
6	15	2
7	25	12
8	17	4
9	0	2

Sample concordance correlation coefficient (p_c): 0.0456

As can be seen from the above table and confirmed by the sample correlation coefficient (p_c), the two approaches based on different primers, and using the same samples analyzed at the same time, produce <u>different</u> values.

Taking into consideration the different values obtained, as seen in Table 2 below, the test sensitivity and specificity are <u>not</u> the same when different primers are used:

Table 2

Sensitivity and specificity of FL-DNA analysis with "claimed" and "new" primers.

Cut-off (ng)	Sensitivity(%)		Specificity(%)	
	Claimed	New	Claimed	New
10	90	80	30	40
15	80	50	70	70
20	70	10	80	100
25	60	10	80	100
30	60	10	90	100

Methods

DNA extraction

Genomic DNA was extracted from 10-20 mg of feces obtained from the spiral groove of the commercially available immunochemical Fecal Occult Blood Test (FOBT (iFOBT OC-Sensor, Alfa Wassermann).

1) One milliliter of TE buffer is added to a 1.5-ml test tube and used to wash the stool attached to the spiral groove.
2) Centrifuge for 15 minutes at 5,000 g. Transfer the supernatant to a clean sterile test tube and add 155 µl of ammonium acetate 7.5 M and 930 µl of ethanol 100%. Mix and centrifuge for 15 minutes at 5,000 g.
3) Extract DNA from the pellet using QIAmp DNA stool kit.

Amplification and FL-DNA analysis is performed in the same way as described in the patent application, the final result is normalized to the data obtained from 4 gr of stool multiplying the results by 3. This value was obtained by evaluating the results of FL-DNA obtained from 4 gr and

10-20 mg of stool in the same samples. A similar normalization value could be obtained using the same approach and quantities other than the standard 4 gr.

Fluorescence long DNA (FL-DNA) analysis

The FL-DNA analysis using the primer described in the claims was performed as previously described in the patent application.

For the new set of primers the p53 sequences were as follows: exon 5: 5A _FAM-CAA CTC TGT CTC CTT CCT CTT CC and 5B_ AAC CAG CCC TGT CGT CTC T; exon 6: 6A_CAG GCC TCT GAT TCC TCA CT and 6B_HEX-CTT AAC CCC TCC TCC CAG AG; exon 7: 7A _FAM-TCA TCT TGG GCC TGT GTT ATC and 7B _TGG AAG AAA TCG GTA AGA GGT G; exon 8: 8A GGG ACA GGT AGG ACC TGA TTT and 8B_HEX-TAA CTG CAC CCT TGG TCT CC.

APC sequences were as follows: fragment 1: 1A_CCC TAG AAC CAA ATC CAG CA and 1B_ HEX-CAT TC ACT GCA TGG TTC AC; fragment 2: 2A_ FAM- GTG AAC CAT GCA GTG GAA TG and 2B_CAC TCA GGC TGG ATG AAC AA; fragment 3: 3A_AAG AAG CTC TGC TGC CCA TA and 3B: HEX- GTG AAC CAT GCA GTG GAA TG; fragment 4: 4A_ FAM- GTC AAT ACC CAG CCG ACC TA and 4B_ GTC AAT ACC CAG CCG ACC TA.

The p53 exons 5 to 8 and fragments 1 to 4 of APC were amplified in a final volume of 25 ml containing 2 ml of stool DNA, 0.4 mM of each primer, 200 mM of deoxynucleotide (Takara Bio Inc), 1X reaction buffer with 3.5 mM MgCl2 (Qiagen), and 1 U of Taq polymerase (Qiagen). The reaction mixture was subjected to 32 cycles: 60 s at 94°C and then 60

s at 58°C f followed by incubation at 72°C for 60 s. for all fragments, Primers used were end-labeled with fluorochromes provided by Applied Bioystems.

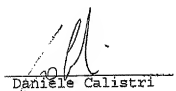
DNA from each sample was quantified on a standard curve of genomic DNA (1, 2, 5, 10 and 20 ng) normalized to 100, and expressed as nanograms.

Electrophoresis was carried out using a 3100 Avant Genetic Analyzer (Applied Biosystems) equipped with GeneScan Analysis 3.7. The final FL-DNA value was obtained by analyzing the fluorescence intensity of each sample-specific PCR product. The quantification of each sample was calculated by reference to a standard curve (1, 2, 5, 10, and 20 ng) of genomic DNA and expressed as nanograms.

The DNA samples were obtained from a small amount of feces (10-15 mg) and for this reason the FL-DNA value obtained using the claimed primers was normalized to the data obtained from 4 gr of stool multiplying the results by 3.

Upon reviewing the results, it is believed to be apparent that not all primers would be considered equivalent or perform the same as suggested by the Official Action.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.



Daniele Calistri

02/12/2010

Date